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(54) Title: PRODUCTION OF RECOMBINANT POLYPEPTIDES BY BOVINE SPECIES AND TRANSGENIC METHODS

(57) Abstract

A transgene for producing recombinant polypeptides in the milk of transgenic bovine species comprises at least one expression regulation sequence, a secretory DNA sequence encoding a secretory signal sequence which is functional in mammary secretory cells of the bovine species and a recombinant DNA sequence encoding the recombinant polypeptide. Also included are methods for producing transgenic bovine species. The method includes introducing the above transgene into an embryonal target cell of a bovine species, transplanting the trangenic embryonic target cell formed thereby into a recipient bovine parent and indentifying at least one female offspring which is capable of producing the recombinant polypeptide in its milk. The invention also includes transgenic bovine species as well as the milk from such transgenic bovine species. Methods are also provided for producing transgenic non-human mammals having a desirable phenotype. The method comprises first methylating a transgene followed by introduction into fertilized oocytes. The oocytes are then cultured to form pre-implantation embryons. Thereafter, at least one cell is removed from each of the pre-implantation embryons and the DNA digested with a restriction endonuclease capable of cleaving the methylated transgene but incapable of cleaving the unmethylated form of the transgene.

Mammary secretion

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PRODUCTION OF RECOMBINANT POLYPEPTIDES BY BOVINE SPECIES AND TRANSGENIC METHODS

Field of the Invention

The invention relates to the production of recombinant polypeptides by transgenic bovine species and to methods for producing transgenic non-human mammals having a desired phenotype.

Background of the Invention

There is a plethora of literature relating to the expression of heterologous genes in lower organisms such as unicellular bacteria, yeast and filamentous fungi, and in higher cell types such as mammalian cells. There are also numerous reports on the production of transgenic animals, most of which relate to the production of transgenic mice. See, e.g., U.S. Pat. No. 4,736,866 (transgenic mice containing activated oncogene); Andres, A., et al. (1987) Proc. Natl. Acad. Sci. USA 84:1299-1303 (HA-RAS oncogene under control of whey acid protein promoter); Schoenberger, C.A., et al.

(1987) Experientia 43:644 and (1988) EMBO J. 7:169-175 (C-myc oncogene under control of whey acid protein promoter); and Muller, W.J., et al. (1988) Cell 54:105-115 (C-myc oncogene under control of the mouse mammary tumor virus promoter). Several laboratories have also reported the production of transgenic porcine species (Miller, K.F., et al. (1989) J. Endocrin. 120:481-488 (expression of human or bovine growth hormone gene in transgenic swine); Vize, P.D., et al. (1988) J. Cell Sci. 90:295-300 (porcine growth hormone fusion gene in transgenic pigs); and Ebert, K. et al. (1988) Mol. Endocrin. 2:277-283 (MMLV-rat somatotropin fusion gene in transgenic pigs)), transgenic sheep (Nancarrow, et al. (1987) Theriogenology 27:263 (transgenic sheep containing bovine growth hormone gene) Clark, A.J. et al. (1989) Bio/Technology 7:487-482 and Simons, J., et al. (1988) Bio/Technology 6:179-183 (human factor IX and α-1 antitrypsin CONA in ovine species), and rabbit (Hanover, S.V., et al. (1987) Deutche Tierarztliche Wochenschrift 94,:476-478 (production of transgenic rabbits by injection of uteroglobin-promoter-CAT fusion gene into fertilized rabbit oocytes). A number of reports have also suggested the production of transgenic cattle (Wagner, et al. (1984) Theriogenology 21:29-44) with one reporting some progress in microinjection techniques (Lohse, J.K., et al. (1985) Theriogenology 23:205). However, little, if any, success has been achieved in producing transgenic cows. Scientific articles which clearly demonstrate the actual production of a transgenic cow capable of producing a heterologous protein are presently unknown. This, despite the statements that one transgenic cow was produced in Canada which expressed human β-interferon (Van Brunt, J. (1988) Bio/Technology 6:1149-1155) and that transient expression of human α-fetoprotein in liver and blood was obtained on one occasion (Church, R.B. (1986) Biotechnology News Watch 6 (15), 4). One reference

reports that bovine papilloma virus was apparently integrated but not expressed in a transgenic cow (Roschlau, et al. (1988) Arch. Tierz., Berlin 31:3-8). A recent article has summarized the genetic engineering of livestock. (Pursel, V.G. et al. (1989) Science 244:1281-1288).

A number of laboratories have reported tissue-specific expression of DNA encoding various proteins in the mammary gland or the production of various proteins in the milk of transgenic mice and sheep. For example, Simmons, J.P., et al. (1987) Nature 328:530-532 report the microinjection of a 16.2 kb genomic fragment encoding β-lactoglobulin (BLG) including 4 kb of 5' sequence, 4.9 kb of the BLG transcription unit and 7.3 kb of 3' flanking sequence into fertilized mouse eggs. According to these authors, the sheep BLG was expressed in mammary tissue and produced BLG in the milk of the transgenic mice at concentrations ranging from about 3.0 to about 23 mg/ml. When, however, cDNA encoding human factor IX or human α1-antitrypsin was inserted into the 5' untranslated region of the BLG gene and microinjected into sheep (Simmons, J.P., et al. (1988) Bio/Technology 6:179-183) the production of factor IX or α1-antitrypsin was significantly reduced (25ng/ml for factor IX and 10mg/ml for α1-antitrypsin; see Clark, A.J., et al. (1989) Bio/Technology 7:487-492).

In a similar approach, a 14 kb genomic clone containing the entire 7.5 kb rat β-casein together with 3.5 kb of 5' and 3.0 kb of 3' flanking DNA was reportedly microinjected into fertilized mouse oocytes. Lee, et al. (1988) Nucl. Acids Res. 16:1027-1041. Yet, in this case, the level of expression of the rat β-transgene in the lactating mammary gland of transgenic mice was

reported to be at a level of 0.01-1% of the endogenous mouse β-casein gene.

Human tissue plasminogen activator (t-PA) reportedly was produced in transgenic mouse milk at the levels between 0.2 and about 0.4μg/ml when a cDNA encoding a human t-PA with its endogenous secretion sequence was expressed under control of a 2.6 kb 5' sequence of the murine whey acid protein gene. Gordon, K., et al. (1987) Bio/Technology 5:1183-1187. Subsequent experiments using the same or similar construction reportedly produced t-PA in different mouse lines arranging from less than 20ng of t-PA per ml of milk to about 50μg/ml. Pittius, C.W., et al. (1988) Proc. Natl. Acad. Sci. USA 85:5874-5878.

U.S. Patent No. 4,873,316 issued October 10, 1989, discloses the use of 9 kb of 5' sequence from the bovine αS1 casein gene including the casein signal peptide and several casein codons fused to a mature t-PA sequence. The transgenic mice obtained with this construct reportedly produced about 0.2-0.5μg/ml of a t-PA fusion protein in their milk.

In addition, a number of patent publications purportedly describe the production of specific proteins in the milk of transgenic mice and sheep. See, e.g. European Patent Publication No. 0 264 166 published April 20, 1988 (hepatitis B surface antigen and t-PA genes under control of the whey acid promoter protein for mammary tissue specific expression in mice); PCT Publication No. W088/00239 published January 14, 1988 (tissue specific expression of a transgene encoding factor IX under control of a whey protein promoter in sheep); PCT Publication No. W088/01648 published March 10, 1988 (transgenic mouse having mammary secretory cells incorporating a recombinant expression system comprising

a bovine α-lactalbumin gene fused to interleukin-2); European Pat. Pub. No. 0 279 582 published August 24, 1988 (tissue-specific expression of chloramphenicol acetyltransferase under control of rat β-casein promoter in transgenic mice); and PCT Pub. No. W088/10118 published December 29, 1988 (transgenic mice and sheep containing transgene encoding bovine αS1 casein promoter and signal sequence fused to t-PA).

Given the state of the transgenic art, it is apparent that a need exists for methods which enable the efficient production of transgenic mammals, especially transgenic mammals other than transgenic mice.

Further, it is apparent that a need exists for methods for producing transgenic bovine species which are capable of producing recombinant polypeptides such as human milk proteins and human serum proteins in the milk of such transgenic mammals.

Accordingly, it is an object herein to provide methods for detecting the transgenesis of fertilized oocytes prior to implantation.

In addition, it is an object herein to provide transgenic bovine species which are capable of producing recombinant polypeptides which are maintained intracellularly or are secreted extracellularly.

It is also an object herein to provide transgenic bovine species which are capable of producing recombinant polypeptides such as human milk proteins and human serum proteins in the milk of such transgenic animals.

Further, it is an object herein to provide milk from a transgenic bovine species containing such recombinant polypeptides.

Still further, it is an object herein to provide food formulations supplemented with recombinant polypeptides from such transgenic milk such as human infant formula supplemented with human lactoferrin.

Further, it is an object herein to provide transgenes which are capable of directing the production of recombinant polypeptides in the milk of transgenic bovine species.

The references discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by priority based on earlier filed applications.

Summary of the Invention

In accordance with the above objects, the invention includes transgenes for producing recombinant polypeptides in the milk of transgenic bovine species. The production of such transgenic bovine milk containing one or more recombinant polypeptides is desirable since it provides a matrix wherein little or no purification is necessary for human consumption. The transgene comprises a secretory DNA sequence encoding a secretory signal sequence which is functional in mammary secretory cells of the bovine species of interest and a recombinant DNA sequence encoding the recombinant polypeptide. These sequences are operably linked to form a secretory-recombinant DNA sequence. At least one expression regulation sequence, functional in the mammary secretory cells of the bovine species, is operably linked to the secretory-recombinant DNA sequence. The transgene so constructed is capable of directing the expression of the secretory-recombinant DNA sequence in mammary secretory cells of bovine

species containing the transgene. Such expression produces a form of recombinant polypeptide which is secreted from the mammary secretory cells into the milk of the transgenic bovine species.

In addition, the invention includes methods for producing such transgenic bovine species. The method includes introducing the above transgene into an embryonal target cell of a bovine species, transplanting the transgenic embryonic target cell formed thereby into a recipient bovine parent and identifying at least one female offspring which is capable of producing the recombinant polypeptide in its milk.

The invention also includes transgenic bovine species capable of producing recombinant polypeptides in the milk of lactating females of said species, the milk from such transgenic bovine species containing such recombinant polypeptides and food formulations containing the transgenic milk in liquid or dried form, as well as food formulations supplemented with one or more recombinant polypeptides from such transgenic milk.

In addition to the foregoing, the invention includes transgenes and transgenic bovine species containing transgenes that are capable of producing a recombinant polypeptide. Such transgenes are similar to the aforementioned transgenes for milk secretion and are characterized by having an expression regulation sequence which targets the expression of the DNA encoding the recombinant polypeptide to a particular cell or tissue type, e.g. expression of human serum albumin in the liver of a transgenic bovine species. When the recombinant polypeptide is to be secreted from such targeted cells or tissues, a secretory DNA sequence encoding a secretory signal sequence functional in the

particular targeted cell or tissue is operably linked to the recombinant DNA sequence encoding the recombinant polypeptide, e.g. secretion of human serum albumin from bovine liver into the bovine circulatory system.

Further, the invention includes methods for producing transgenic non-human mammals having a desirable phenotype. The method comprises first causing the methylation of a transgene capable of conferring the desirable phenotype when incorporated into the cells of a transgenic non-human animal, e.g., by transforming an appropriate bacterium, such as E. coli MM 294, with a plasmid containing the transgene. The methylated transgene is then excised and introduced into fertilized oocytes of the non-human animal to permit integration into the genome. The oocytes are then cultured to form pre-implantation embryos thereby replicating the genome of each of the fertilized oocytes. Thereafter, at least one cell is removed from each of the pre-implantation embryos and treated to release the DNA contained therein. Each of the released DNAs are then digested with a restriction endonuclease capable of cleaving the methylated transgene but incapable of cleaving the unmethylated form of the transgene formed after integration into and replication of the genomic DNA. Those pre-implantation embryos which have integrated the transgene contain DNA which is resistant to cleavage by the restriction endonuclease in the region containing the transgene. This resistance to digestion, which can be detected by electrophoresis of the digest after PCR amplification of the DNA and hybridization with a labelled probe for the transgene, facilitates the identification of successful transgenesis.

The invention also includes a method to produce a population of transgenic offspring having the same genotype. This method utilizes a specific embodiment

of the above method for detecting early transgenesis. In this method, a methylated transgene is introduced into fertilized oocytes which are cultured to pre-implantation embryos. Thereafter, each pre-implantation embryo is divided to form first and second hemi-embryos. Each of the first hemi-embryos are then analyzed for transgenesis as described above. After identifying successful transgenesis in at least one first hemi-embryo, the second untreated hemi-embryo which contains the integrated transgene, is cloned to form a multiplicity of clonal transgenic blastocysts or hemi-blastocysts, each of which have the same genotype. The transgenic embryos are thereafter transplanted into one or more recipient female parents to produce a population of transgenic non-human mammals having the same genotype.

Brief Description of the Drawings

The accompanying drawings, which are incorporated in and form a part of the specification, illustrate embodiments of the present invention and, together with the description, serve to explain the principles of the invention. In the drawings:

Fig. 1 depicts the DNA (Seq. ID No.: 1) and amino acid (Seq. ID No.: 2) sequence for a human lactoferrin clone derived from a human mammary cDNA library as described herein except that the sequence between nucleotides 1557-1791 and 2050-2119 corresponds to the previously published sequence (Rado et al. (1987) Blood 70:989-993).

Fig. 2 depicts the complete DNA (Seq. ID No.: 3) and amino acid (Seq. ID No.: 4) sequence of human lactoferrin including 5' and 3' untranslated sequence as well as the complete human lactoferrin signal sequence.

Fig. 3 is a restriction map of a clone of a 5'-flanking region of bovine αS1 casein gene.

Fig. 4 is a restriction map of a clone of a 3'-flanking region of bovine αS1 casein gene.

Figs. 5A, 5B and 5C depict the construction of pSI3'5'CAT and pSI5'CAT.

Fig. 6 depicts pMH-1.

Figs. 7A through 7F depict the construction of expression vectors containing sequences encoding human lactoferrin.

Fig. 8 depicts the genome of human serum albumin, the fragments used to generate transgenic mice contained in this genomic DNA and the identification of the fragment sizes which would be obtained upon the digestion of genomic DNA from a transgenic mouse with the restriction enzymes BstE-II and Nco-I or with Nco-I and Hindi-III.

Fig. 9 depicts an alternate pathway for the construction of a transgene of the invention encoding human lactoferrin.

Fig. 10 depicts the construction of a plasmid pPC containing a transgene encoding Protein C.

Fig. 11 depicts the DNA sequence for a hybrid intervening sequence used in a preferred embodiment of the invention. This hybrid sequence comprises a 5' portion from an intervening sequence of bovine αS1 casein and a 3' portion from an intervening sequence of an IgG intervening sequence. The juncture of the 5' and 3' portion is the HindIII site shown.

Fig. 12A is a restriction map of a bovine αS1 casein promoter hLF cDNA transgene.

Fig. 12B shows a Southern blot analysis of DNA isolated from various bovine and murine tissues using an hLF cDNA probe.

Fig. 13 depicts restriction maps of hLF genomic clones 13.1 and 13.2.

Fig. 14 depicts the BamHI fragment from genomic hLF subcloned into plasmid pUC19.

Fig. 15A depicts a restriction map of the 8hLFgen9k or 16hLFgen9k construct containing the 8 or 16 kb αS1 casein promoter, a ClaI-ApaI synthetic linker and the 9 kb (i.e., 8.9 kb) ApaI-SalI genomic hLF fragment.

Fig. 15B depicts the DNA sequence of the ClaI-ApaI synthetic sequence shown in Fig. 15A.

Fig. 15C depicts the IVS and the structure of exon 1 and part of exon 2 of the genomic hLF construct shown in Fig. 15A through Fig. 17.

Fig. 16 depicts the coinjection of the NotI-SalI fragment from the 8hLFgen9k or 16hLFgen9k construct (as shown in Fig. 15A) with the 3' ClaI fragment of genomic hLF.

Fig. 17 depicts the generation of a genomic 8hLF transgene by linking the NotI-MluI fragment from the 8hLFgen9k construction (shown in Fig. 15A), the MluI-ClaI fragment from clone 13.2 depicted in Fig. 13 and a ClaI-NotI linker. Fig. 17 also depicts the DNA sequence of the ClaI-NotI linker.

Figs. 18-20 depict the generation of the βLG-hLFgen and βLG-hLFgen37 constructs.

Fig. 21 depicts the design of the 16,8hLZ expression vector.

Fig. 22 depicts the design of the 16,8hLZ3 expression vector.

Fig. 23A-23E depict the pathway for the construction of plasmid p16,8hLZ.

Fig. 24 depicts a comparison between the DNA of bovine βLG and sheep βLG. The top sequence represents the bovine sequence.

Fig. 25 shows the linker GP 278/279.

Fig. 26 depicts the p16,8A hLZ3 expression vector.

Fig. 27 depicts the 16,A hLZ3 expression vector.

Detailed Description of the Invention

The "non-human mammals" of the invention comprise all non-human mammals capable of producing a "transgenic non-human mammal" having a "desirable phenotype". Such mammals include non-human primates, murine species, bovine species, canine species, etc. Preferred non-human animals include bovine, porcine and ovine species, most preferably bovine species.

Desirable phenotypes for transgenic non-human mammals include, but are not limited to, the production of recombinant polypeptides in the milk of female transgenic non-human mammals, the production of animal models for the study of disease, the production of

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Desirable phenotypes for transgenic non-human mammals include, but are not limited to, the production of recombinant polypeptides in the milk of female transgenic non-human mammals, the production of animal models for the study of disease, the production of

animals with higher resistance to disease (e.g. diseases of the mammary gland such as mastitis) and the production of recombinant polypeptides in the blood, urine or other suitable body fluid or tissue of the animal. In the preferred embodiments, transgenic bovine species are disclosed which are capable of producing recombinant human lactoferrin, human serum albumin and human Protein C in the milk of lactating females or human serum albumin in the liver of the transgenic animal.

The transgenic non-human mammals of the invention are produced by introducing a "transgene" into an embryonal target cell of the animal of choice. In one aspect of the invention, a transgene is a DNA sequence which is capable of producing a desirable phenotype when contained in the genome of cells of a transgenic non-human mammal. In specific embodiments, the transgene comprises a "recombinant DNA sequence" encoding a "recombinant polypeptide". In such cases, the transgene is capable of being expressed to produce the recombinant polypeptide.

As used herein, a "recombinant polypeptide" (or the recombinant DNA sequence encoding the same) is either a "heterologous polypeptide" or a "homologous polypeptide". Heterologous polypeptides are polypeptides which are not normally produced by the transgenic animal. Examples of heterologous polypeptides include human milk proteins such as lactoferrin, lysozyme, secreted immunoglobulins, lactalbumin, bile salt-stimulated lipase, etc., human serum proteins such as albumin, immunoglobulins, Factor VIII, Factor IX, protein C, etc. and industrial enzymes such as proteases, lipases, chitinases, and liginases from procaryotic and eucaryotic sources. The

recombinant DNA sequences include genomic and cDNA sequences encoding the recombinant polypeptide.

When recombinant DNA sequences encoding a heterologous polypeptide are used, the transgene may be integrated in a random manner into the genome of the species used for transgenesis. As disclosed in the Examples, transgenes encoding human lactoferrin, human serum albumin and human Protein C in conjunction with a αS1 casein secretory signal sequence under control of αS1 casein expression regulation sequences are designed to produce and secrete these heterologous polypeptides from the mammary gland of a lactating transgenic mammal into its milk.

As used herein, a homologous polypeptide is one which is endogenous to the particular transgenic species. Examples of endogenous polypeptides from bovine species include bovine milk proteins such as αS1, αS2, β- and κ-casein, β-lactoglobulin lactoferrin, lysozyme, cholesterol hydrolase, serum proteins such as serum albumin and proteinaceous hormones such as growth hormones. When recombinant DNA sequences encoding a homologous polypeptide are used, the transgene is preferably integrated in a random manner into the genome of the species used for transgenesis. Such random integration results in a transgenic animal which contains not only the transgene encoding the endogenous polypeptide but also the corresponding endogenous genomic DNA sequence. Accordingly, such transgenic non-human mammals are readily characterized by an increase in the copy number of genes encoding the endogenous polypeptide. Further, the transgene will generally be located at a position which is different from the endogenous gene.

When DNA encoding a homologous polypeptide is expressed, for example, in bovine species, the transgenic animal is characterized by an increase in the amount of the homologous polypeptide in either the endogenous tissue or fluid in which it is normally found and/or by its presence in a tissue and/or body fluid which either does not normally contain the homologous polypeptide or produces it at significantly lower levels.

Thus, for example, bovine cholesterol hydrolase is normally present in the colostrum for about the first 15-20 days of lactation. This naturally occurring endogenous polypeptide increases calf weight. This protein, however, is also a homologous polypeptide when, for example, its expression in mammary secretory cells is placed under the control of expression regulation sequences, such as those obtained from bovine casein genes, which facilitate the expression of the homologous polypeptide beyond the lactation period that it is normally present. Thus, according to one aspect of the invention, bovine cholesterol hydrolase expression is maintained in transgenic bovine milk by placing the expression of cholesterol hydrolase recombinant DNA (either cDNA or genomic) under the control of bovine αS1 casein expression regulation sequences. When a genomic recombinant DNA is used, it is engineered such that it has appropriate restriction sites (e.g. ClaI and SalI) at the 5' and 3' end of the structural gene such that it is capable of being inserted into an appropriate transgene genomic cassette (e.g. p-16 kb, CS which is described in Example 15). Alternatively, a recombinant DNA encoding bovine cholesterol hydrolase derived from cDNA may be placed under control of bovine αS1 casein expression regulation sequence by substituting the human lactoferrin sequences in a plasmid such as p16, 8HLF3 (containing a hybrid intervening sequence) or p16, 8HLF4 (containing a homologous αS1 casein intervening

sequence). When these particular plasmids are used, the cDNA clone is engineered such that it has appropriate ClaI and SalI restriction sites at the ends of the recombinant DNA.

By way of further example, bovine lactoferrin is normally present in only trace amounts in cow's milk. When, however, bovine lactoferrin is expressed under control of other regulatory sequences, for example, obtained from an αS1 casein gene, higher amounts of lactoferrin in the milk of transgenic bovine species are obtained. In another example, a transgene comprising DNA encoding homologous bovine growth hormone is incorporated into the bovine genome to confer superior growth characteristics to the transgenic animal. In other instances, homologous polypeptides include, for example, a polypeptide which normally is maintained intracellularly in a particular species but which is secreted into the milk or other extracellular compartment of the transgenic species, such as the circulatory system.

Each of the heterologous or homologous polypeptides are characterized by specific amino acid and nucleic acid sequences. It is to be understood, however, that such sequences include naturally occurring allelic variations thereof and variants produced by recombinant methods wherein such nucleic acid and polypeptide sequences have been modified by the substitution, insertion and/or deletion of one or more nucleotides in such nucleic acids to cause the substitution, insertion or deletion of one ore more amino acid residues in the recombinant polypeptide.

When expression of the DNA of the transgene is necessary to generate a desired phenotype, e.g. to produce a recombinant polypeptide, the transgene typically

includes at least a 5' and preferably additional 3' "expression regulation sequences" each operably linked to a recombinant or secretory-recombinant DNA as defined hereinafter. Such expression regulation sequences in addition to controlling transcription also contribute to RNA stability and processing, at least to the extent they are also transcribed.

Such expression regulation sequences are chosen to produce tissue-specific or cell type-specific expression of the recombinant or secretory-recombinant DNA. Once a tissue or cell type is chosen for expression, 5' and optional 3' expression regulation sequences are chosen. Generally, such expression regulation sequences are derived from genes that are expressed primarily in the tissue or cell type chosen. Preferably, the genes from which these expression regulation sequences are obtained are expressed substantially only in the tissue or cell type chosen, although secondary expression in other tissue and/or cell types is acceptable if expression of the recombinant DNA in the transgene in such tissue or cell type is not detrimental to the transgenic animal. Particularly preferred expression regulation sequences are those endogenous to the species of animal to be manipulated. However, expression regulation sequences from other species such as those from human genes may also be used. Particularly preferred expression regulation sequences from human genes are human lactoferrin (hLF) sequences. In some instances, the expression regulation sequences and the recombinant DNA sequences (either genomic or CDNA) are from the same species, e.g., each from bovine species or from a human source. In such cases, the expression regulation sequence and the recombinant DNA sequence are homologous to each other. Alteratively, the expression regulation sequences and recombinant DNA sequences (either cDNA or genomic) are obtained from different species, e.g., an

expression regulation sequence from bovine species and a recombinant DNA sequence from a human source). In such cases, the expression regulation and recombinant DNA sequence are heterologous to each other. The following defines expression regulation sequences from endogenous genes. Such definitions are also applicable to expression regulation sequences from non-endogenous, heterologous genes.

In general, the 5' expression regulation sequence includes the transcribed portion of the endogenous gene upstream from the translation initiation sequence (the 5' untranslated region or 5' UTR) and those flanking sequences upstream therefrom which comprise a functional promoter. As used herein, a "functional promoter" includes those necessary untranscribed DNA sequences which direct the binding of RNA polymerase to the endogenous gene to promote transcription. Such sequences typically comprise a TATA sequence or box located generally about 25 to 30 nucleotides from the transcription initiation site. The TATA box is also sometimes referred to the proximal signal. In many instances, the promoter further comprises one or more distal signals located upstream from the proximal signal (TATA box) which are necessary to initiate transcription. Such promoter sequences are generally contained within the first 100 to 200 nucleotides located upstream from the transcription initiation site, but may extend up to 500 to 600 nucleotides from the transcription initiation site. Such sequences are either readily apparent to those skilled in the art or readily identifiable by standard methods. Such promoter sequences alone or in combination with the 5' untranslated region are referred to herein as "proximal 5' expression regulation sequences".

In addition to such proximal 5' expression regulation sequences, it is preferred that additional 5' flanking sequences (referred to herein as "distal 5' expression regulation sequences") also be included in the transgene. Such distal 5' expression regulation sequences are believed to contain one or more enhancer and/or other sequences which facilitate expression of the endogenous gene and as a consequence facilitate the expression of the recombinant or secretory-recombinant DNA sequence operably linked to the distal and proximal 5' expression regulation sequences. The amount of distal 5' expression regulation sequence depends upon the endogenous gene from which the expression regulation sequences are derived. In general, however, such sequences comprise 5' flanking regions of approximately 1 kb, more preferably 16 kb and most preferably about 30 kb of 5' flanking sequence. The determination of the optimal amount of distal 5' expression regulation sequence used from any particular endogenous gene is readily determined by varying the amount of distal 5' expression regulation sequence to obtain maximal expression. In general, the distal 5' expression regulation sequence will not be so large as to extend into an adjacent gene and will not include DNA sequences which adversely effect the level of transgene expression.

In addition, it is preferred that 3' expression regulation sequences also be included to supplement tissue or cell-type specific expression. Such 3' expression regulation sequences include 3' proximal and 3' distal expression regulation sequences from an appropriate endogenous gene. The 3' proximal expression regulation sequences include transcribed but untranslated DNA positioned downstream from the translation stop signal in the recombinant DNA sequence (also referred to as the 3' untranslated region or 3'

UTR). Such sequences generally terminate at a polyadenylation sequence (either from the endogenous gene or from other sources such as SV40) and sequences that may affect RNA stability. Generally, 3' UTR's comprise about 100 to 500 nucleotides downstream from the translation stop signal in the gene from which the 3' regulation sequence is derived. Distal 3' expression regulation sequences include flanking DNA sequences downstream from the proximal 3' expression regulation sequence. Some of these distal sequences are transcribed, but do not form part of the mRNA while other sequences in this distal 3' expression regulation sequence are not transcribed at all. Such distal 3' expression regulation sequences are believed to contain enhancer and/or other sequences which enhance expression. Such sequences are believed to be necessary for efficient polydenylation and contain transcription termination sequences Preferably, such sequences comprise about 2 kb, more preferably 8 kb and most preferably about 15 kb of 3' flanking sequence.

A preferred 3' flanking sequence is the 3' flanking sequence of the human lactoferrin (hLF) gene. Transgenic animals containing transgenes that include about 9 kb of hLF 3' flanking sequences show enhanced expression of recombinant polypeptides in milk compared to animals containing transgenes that include 1 kb or less of hLF 3' flanking sequence, due to an enhancer or other enhancing sequence located in this region. Usually the human lactoferrin 3' flanking sequence will be at least 1 kb in length up to about 9 kb in length or longer, typically 3 to 7 kb, more typically 4 to 5 kb. It will also be possible, and sometimes desirable, to use standard methods (e.g., deletion analysis) to identify regions contained within the 9 kb 3' flanking sequence that enhance mammary gland expression of recombinant polypeptides. These enhancers or enhancing sequences can

be isolated and used in combination with various amounts of homologous or heterologous sequences. Typically the enhancing sequences can range in length from about 50 basepairs to about 2 kb, more typically from about 100 basepairs to about 500 basepairs.

It will often be desirable to use a transgene having a 5' expression regulation sequence and a 3' flanking sequence that originate from the same gene. In a preferred embodiment, the 5' expression regulation sequence and 3' flanking sequence are from the bovine αS1-casein gene.

In an alternative embodiment a genomic sequence, such as a human genomic clone or clones, can be introduced into an animal to produce a transgenic animal containing a transgene that has the sequence of the human gene, including all or part of the 5' expression regulation sequences, coding sequences, introns, and 3' untranslated and flanking sequences. In a preferred embodiment, the human lactoferrin genomic sequence is used in its entirety, but various components can be substituted with components from other mammary gland specific genes.

Although the use of both 5' and 3' expression regulation sequences are preferred, in some embodiments of the invention, endogenous 3' regulation sequences are not used. In such cases, the 3' proximal expression regulation sequences normally associated with the genomic DNA encoded by the recombinant DNA sequence are used to direct polyadenylation. In addition, distal 3' regulation sequences from the genomic DNA encoding the recombinant polypeptide may also be employed preferably in the same amounts as set forth for endogenous 3' expression regulation sequences. In such cases, it is to be understood that the recombinant polypeptide

encoded by the transgene may comprise either genomic DNA or a double stranded DNA derived from cDNA. As with the 5' expression regulation sequences, the optimal amount of 3' expression regulation sequence may be readily determined by varying the amount of 3' flanking sequence to obtain maximal expression of the recombinant polypeptide. In general, the distal 3' regulation sequence, be it from an endogenous gene or a heterologous gene, will not extend into the adjacent gene from which is derived and will exclude any sequences which adversely effect the level of transgene expression.

Examples of expression regulation sequences are provided in Table I.

TABLE 1

Expression Regulation Sequence	Tissue Specificity	Animal Species
16 kb of bovine αS1 casein 5' to structural gene and 8 kb 3' to structural gene	Mammary secretory cells	bovine
≈15 kb 5' to albumin gene	Liver	murine
≈15 kb 5' to α-actin gene	Muscle	murine
≈15 kb upstream of protamine gene	Spermatids	murine

In addition to the 5' and 3' expression regulation sequences and the recombinant DNA (either genomic or derived from cDNA) the transgenes of the invention preferably also comprise a "recombinant intervening sequence" which interrupts the transcribed but untranslated 5' region of the transgene. Such intervening sequences can be derived, for example, from

bovine αS1 casein and from human lactoferrin. Such sequences as used herein are "homologous recombinant intervening sequences" in that the 5' and 3' RNA splice signals in such recombinant intervening sequences are those normally found in an intervening sequence from an endogenous or heterologous gene. Recombinant intervening sequences may, however, also comprise a "hybrid intervening sequence". Such hybrid intervening sequences comprise a 5' RNA splice signal and 3' RNA splice signal from intervening sequences from different sources. In some aspects of the invention, such hybrid intervening sequences comprise at least one "permissive RNA splice sequence". As used herein, a permissive RNA splice signal is an RNA splice signal sequence, preferably a 3' RNA splice signal, from an intron contained within a repertoire of germ line DNA segments which undergo rearrangement during cell differentiation. Examples of such gene repertoires include the immunoglobulin super gene family, including the immunoglobulins and T-cell antigen receptors as well as the repertoire of the major histocompatibility complex (MHC) genes and others. Particularly preferred permissive splice sequences are those obtained from the immunoglobulin repertoire, preferably of the IgG class, and more preferably those 3' splice signal sequences associated with the J-C segment rearrangement of the Ig heavy and light chain, most preferably the heavy chain. A particularly preferred permissive splice sequence comprises that portion of the sequence as shown downstream of the HindIII site in Fig. 11. A particularly preferred hybrid intervening sequence comprises the entire sequence shown in Fig. 11 which includes a 5' portion of an intervening sequence from bovine αS1 casein and a 3' sequence portion of an IgG heavy chain intervening sequence.

Such hybrid intervening sequences containing permissive RNA splice signals are preferably used when the recombinant DNA corresponds to a cDNA sequence. As indicated in the Examples, when 16 kb of 5' expression regulation sequence from the αS1 casein gene was used in conjunction with an αS1 casein-IgG hybrid intervening sequence to express human lactoferrin cDNA operably linked to the αS1 casein secretory signal sequence a transgenic mouse was obtained which produced approximately 1330 μg/ml of hLF in the transgenic milk. This amount of recombinant polypeptide far exceeds the previously reported amounts for production of various protein in transgenic mouse milk of generally less than 10 μg/ml and in one case approximately 50 μg/ml. It also exceeds the maximum of 8μg/ml of hLF produced herein when the same transgene was used that contained a homologous bovine intervening sequence rather than the hybrid intervening sequence.

However, such hybrid intervening sequences are not limited to transgenes utilizing cDNA sequence. Rather, hybrid intervening sequences are also useful when the recombinant polypeptide is encoded by a genomic sequence. Based on the results obtained with the cDNA recombinant DNA and the general expectation that genomic DNA sequences express at higher levels than sequences derived from cDNA, it is expected that such hybrid intervening sequences used in conjunction with genomic recombinant DNA will further enhance expression levels above that which would otherwise be obtained with genomic sequence alone.

Based on the foregoing, it is apparent that preferred transgenes include large amounts of 5' and 3' expression regulation sequences. Further, the recombinant DNA is preferably derived from genomic clones which may be tens to hundreds of kilobases in length. Based on the

present technology for cloning and manipulating DNA, the construction and microinjection of transgenes is practically limited to linearized DNA having a length not greater than about 50 kb. However, the transgenes of the invention, especially those having a length greater than about 50 kb, may be readily generated by introducing two or more overlapping fragments of the desired transgene into an embryonal target cell. When so introduced, the overlapping fragments undergo homologous recombination which results in integration of the fully reconstituted transgene in the genome of the target cell. In general, it is preferred that such overlapping transgene fragments have 100% homology in those regions which overlap. However, lower sequence homology may be tolerated provided efficient homologous recombination occurs. If non-homology does exist between the homologous sequence portions, it is preferred that the non-homology not be spread throughout the homologous sequence portion but rather be located in discrete areas. Although as few as 14 base pairs at 100% homology are sufficient for homologous recombination in mammalian cells (Rubnitz, J. and Subramani, S. (1984) Mol. Cell. Biol. 4:2253-2258), longer homologous sequence portions are preferred, e.g. 500bp, more preferably 1000bp, next most preferably 2000bp and most preferably greater than 2000bp for each homologous sequence portion.

As indicated in the examples, three overlapping fragments of the human serum albumin gene were microinjected into the pronuclei of mouse zygotes in approximately equal molar portions. These fragments successfully recombined and integrated into the mouse genome as confirmed by analysis of the integrated DNA by Southern blotting procedures and by detection of RNA transcript and human serum albumin in the serum of the transgenic mouse. Although the transgene so generated

has a unit length of 38 kb, there is no known practical limit to the size of the transgene which may be formed using larger and/or greater numbers of overlapping transgene fragments. In particular, it is expected that transgenes may be formed by this approach having lengths between about 50 to 1000 kb and more preferably between 50 and 500 kb. Further, the use of homologous recombination of overlapping fragments is expected to be fruitful in the generation of larger transgenic animals, such as transgenic bovine species, containing transgenes incorporating recombinant DNA comprising genomic DNA which otherwise could not be incorporated into a pronucleus to form a transgenic animal. Such genomic transgenes are expected to produce higher expression levels in transgenic cows as compared to that which is produced by transgenes encoding recombinant cDNA.

When, the ultimate object is to secrete a recombinant polypeptide, a "secretory DNA sequence" encoding a functional secretion signal peptide is also operably linked within the transgene to direct secretion of the recombinant polypeptide from one or more cell types within the transgenic animal. Secretory DNA sequences in general are derived from genes encoding secreted proteins of the same species of the transgenic animal. Such secretory DNA sequences are preferably derived from genes encoding polypeptides secreted from the cell type targeted for tissue-specific expression, e.g. secreted milk proteins for expression in and secretion from mammary secretory cells. Secretory DNA sequences, however, are not limited to such sequences. Secretory DNA sequences from proteins secreted from other cell types within the species of transgenic animal may also be used, e.g., the native signal sequence of a homologous gene encoding a protein secreted other than in the mammary glands. In addition, "heterologous

secretory DNA sequences" which encode signal secretion peptides from species other than the transgenic animals my also be used e.g., human t-PA, human serum albumin human lactoferrin and human lactalbumin and secretion signals from microbial genes encoding secreted polypeptides such as from yeast, filamentous fungi, and bacteria. In general, a secretory DNA sequence may be defined functionally as any DNA sequence which when operably linked to a recombinant DNA sequence encodes a signal peptide which is capable of causing the secretion of the recombinant polypeptide.

In one of the preferred embodiments, a secretory DNA sequence encoding a secretory signal sequence functional in the mammary secretory cells of bovine species is used to cause secretion of recombinant polypeptide from bovine mammary secretory cells. The secretory DNA sequence is operably linked to the recombinant DNA sequence. Examples of such secretory DNA sequences include DNA sequences encoding signal secretion sequences for bovine αS1 casein, murine lactoferrin and human transferrin. The preferred secretory DNA sequence is that encoding the secretory sequence of αS1 casein from bovine species. The use of this secretory DNA sequence is described in more detail in the Examples.

"Operably linked" in the context of linking a secretory DNA sequence to a recombinant DNA sequence means that the secretory DNA sequence (comprising codons encoding the secretory signal peptide sequence) is covalently coupled to the recombinant DNA sequence so that the resultant secretory-recombinant DNA sequence encodes 5' to 3' for the secretory signal sequence and recombinant polypeptide. Accordingly, the reading frame for the secretory sequence and the recombinant DNA sequence must be covalently combined such that an open reading frame exists from the 5' end of the mRNA sequence formed after

transcription and processing of the primary RNA transcript. This open reading frame in the RNA contains a 5' sequence portion encoding the secretory signal peptide and a 3' sequence portion encoding the recombinant polypeptide. When so constructed, the recombinant polypeptide produced upon expression of the secretory-recombinant DNA sequence is of a form which is capable of being secreted from targeted cells which express the DNA sequence. The signal peptide generally is removed in vivo during secretion to produce an extracellular form of the recombinant polypeptide.

In the preferred embodiments of the invention, a secretory-recombinant DNA sequence is expressed predominantly in the mammary secretory cells of transgenic bovine species. Such tissue-specific expression is obtained by operably linking mammary specific expression regulation DNA sequences to the above secretory-recombinant DNA sequence. Such mammary specific regulation sequences include the aforementioned regulation sequences contained in various bovine genes preferentially expressed in the mammary secretory cells of the species. Such mammary specific genes include αS1 casein; αS2-casein; β-casein; K-casein; α-lactalbumin; and β-lactoglobulin. Preferred expression regulation sequences are derived from αS1 casein as described more in detail in the Examples.

In general, the transgenes of the invention that are designed to secrete the recombinant polypeptide into transgenic bovine milk are capable of causing such secretion at levels significantly higher than that previously reported for transgenic mice and sheep. When the recombinant polypeptide is encoded by a recombinant DNA corresponding to, or derived from, cDNA, the molar concentration of the recombinant polypeptide is preferably greater than about 1.0 μM, more preferably

greater than about 100 μM, and most preferably greater than 100 μM. When viewed from the perspective of the level of recombinant polypeptide present in the transgenic milk, the amount of recombinant polypeptide is preferably greater than 50 μg/ml, more preferably greater than about 500 μg/ml and most preferably greater than about 1000 μg/ml (1mg/ml).

When the transgene of the invention encodes a recombinant polypeptide that is encoded by recombinant DNA derived from or corresponding to genomic DNA (or comprised substantially of such genomic sequences, e.g. greater than about 50%, more preferably greater than about 75%, most preferably greater than 90% of the codons encoding the recombinant polypeptide are from genomic sequences), the molar concentrations and protein levels in bovine transgenic milk are the same as for cDNA or higher. In general, the molar concentration of the recombinant polypeptide in such transgenic milk is preferably greater than about 50 μM, more preferably greater than about 150 μM, most preferably greater than about 500 μM. When viewed from the level of protein in the transgenic milk, the levels are preferably greater than about 10 mg/ml, more preferably greater than about 2.5 mg/ml, most preferably greater than 5 mg/ml.

The foregoing molar concentration and protein levels in bovine transgenic milk will vary depending upon the molecular weight of the particular recombinant polypeptide. A particular advantage of producing a recombinant polypeptide in bovine transgenic milk is that relatively large molecular weight polypeptides may be so produced which are otherwise difficult to produce in large quantities in other systems such as prokaryotic expression systems. Although any recombinant polypeptide may be produced in bovine transgenic milk according to the invention, it is generally preferred

that such recombinant polypeptides have a molecular weight greater than about 10,000 Daltons. However, other recombinant polypeptides having molecular weights of greater than 15,000, greater than 20,000 and greater than 60,000 Daltons may also be expressed in transgenic bovine milk. For example, human lysozyme having a molecular weight of 17,000 Daltons and lactoferrin having a molecular weight of 79,000 Daltons may be readily produced in the transgenic milk of bovine species according to the disclosure of the invention. Thus, the recombinant polypeptides of the invention have a wide range of molecular weights.

As a consequence, the foregoing preferred molar concentrations of recombinant polypeptides are adjusted when higher molecular weight recombinant polypeptides are produced. Such adjustment is made by converting the molar concentration to the amount of protein produced and adjusting the molar concentrations so that the recombinant protein level is within the following preferred concentrations.

Most of the previous reports relating to the production of polypeptides in transgenic milk involve transgenic mice. The mouse, however, normally produces between 55 to 80 milligrams of protein per ml of milk. A cow, on the other hand, normally produces between 30 to 34 milligrams of protein per ml. Since exceptionally high levels of recombinant polypeptide production may adversely affect the production of endogenous milk protein and/or have adverse effects upon the mammary secretory gland, it is preferred that the recombinant polypeptide concentration be between about 3 and 50% of the normal bovine milk protein concentration (i.e., between about 1 and 17 milligrams of recombinant polypeptide per ml of transgenic milk), more preferably between 10 to 20% (i.e., between 3 to about 7 milligrams

per ml) and most preferably between 10 and 15% (i.e., between about 3 and 5 milligrams per ml) of the normal amount of protein produced in bovine milk. Such preferred ranges also provide a preferred maximum limit to the aforementioned levels of protein produced in transgenic bovine milk.

The above described linking of various DNA sequences to form the transgene of the invention are performed by standard methods known to those skilled in the art or as described herein. Once the transgene or overlapping homologous fragments encoding the transgene are constructed as described they are used to make transgenic non-human animals.

Methods of introducing transgenes or overlapping transgene fragments into embryonal target cells include microinjection of the transgene into the pronuclei of fertilized oocytes or nuclei of ES cells of the non-human animal. Such methods for murine species are well known to those skilled in the art. Alternatively, the transgene may be introduced into an animal by infection of zygotes with a retrovirus containing the transgene (Jaenisch, R. (1976) Proc. Natl. Acad. Sci. USA 73:1260-1264). The preferred method is microinjection of the fertilized oocyte. In this preferred embodiment, the fertilized oocytes are first microinjected by standard techniques. They are thereafter cultured in vitro until a "pre-implantation embryo" is obtained. Such pre-implantation embryos preferably contain approximately 16 to 150 cells. The 16 to 32 cell stage of an embryo is commonly referred to as a morula. Those pre-implantation embryos containing more than 32 cells are commonly referred to as blastocysts. They are generally characterized as demonstrating the development of a blastocoel cavity typically at the 64 cell stage. Methods for culturing fertilized oocytes to the pre-

implantation stage include those described by Gordon, et al. (1984) Methods in Enzymology 101:414; Hogan, et al. (1986) in Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (for the mouse embryo); and Hammer, et al. (1985) Nature 315:680 (for rabbit and porcine embryos) Gandolfi, et al. (1987) J. Reprod. Fert. 81:23-28; Rexroad, et al. (1988) J. Anim. Sci. 66:947-953 (for ovine embryos) and Eyestone, W.H. et al. (1989) J. Reprod. Fert. 85:715-720; Camous., et al. (1984) J. Reprod. Fert. 72:779-785; and Heyman, Y., et al. (1987) Theriogenology 27:5968 (for bovine embryos). Such pre-implantation embryos are thereafter transferred to an appropriate female by standard methods to permit the birth of a transgenic or chimeric animal depending upon the stage of development when the transgene is introduced. As is well known, mosaic animals can be bred to form true germline transgenic animals.

Since the frequency of transgene incorporation is often low, the detection of transgene integration in the pre-implantation embryo is highly desirable. In one aspect of the invention methods are provided for identifying embryos wherein transgenesis has occurred and which permit implantation of transgenic embryos to form transgenic animals. In this method, one or more cells are removed from the pre-implantation embryo. When equal division is used, the embryo is preferably not cultivated past the morula stage (32 cells). Division of the pre-implantation embryo (reviewed by Williams et al. (1986) Theriogenology 22:521-531) results in two "hemi-embryos" (hemi-morula or hemi-blastocyst) one of which is capable of subsequent development after implantation into the appropriate female to develop in utero to term. Although equal division of the pre-implantation embryo is preferred, it is to be understood that such an embryo may be unequally divided either

intentionally or unintentionally into two hemi-embryos which are not necessarily of equal cell number. Essentially, all that is required is that one of the embryos which is not analyzed as hereinafter described be of sufficient cell number to develop to full term in utero. In a specific embodiment, the hemi-embryo which is not analyzed as described herein, if shown to be transgenic, is used to generate a clonal population of transgenic non-human animals.

One of each of the hemi-embryos formed by division of pre-implantation embryos is analyzed to determine if the transgene has been integrated into the genome of the organism. Each of the other hemi-embryos is maintained for subsequent implantation into a recipient female of the species. A preferred method for detecting transgenesis at this early stage in the embryo's development uses these hemi-embryos in connection with a unique property of the restriction endonuclease Dpn I. This enzyme recognizes the sequence GATC in double-stranded DNA but only when the adenine in each strand within this sequence is methylated at N-6. When using this preferred method, the transgene containing the sequence GATC is methylated prior to microinjection either by transferring the transgene on an appropriate plasmid through a DAM^+ strain of microorganisms such as E. coli MM294 or by directly methylating the transgene with dam methylase. The methylated transgene (preferably without any exogenous sequences such as plasmid vector) is then microinjected into fertilized oocytes (approximately 10 to 500 copies per pronucleus, more preferably 50 to 100 copies per pronucleus). The fertilized oocytes so obtained are cultured in vitro to the pre-implantation stage. During this early growth and cell division phase, the genomic DNA is replicated. Accordingly, those copies of the methylated transgene integrated into the genome of the fertilized oocyte are

unmethylated after replication whereas any non-integrated transgenes which may still exist after replication will remain methylated. (Lacks, S., et al. (1977) J. Mol. Biol. 114:153.) This differential methylation pattern for integrated versus non-integrated transgene permits the identification of which fertilized oocytes have integrated the transgene into the genome.

The identification of the pre-implantation embryos containing the integrated transgene is achieved by analyzing the DNA from each of the hemi-embryos. Such DNA is typically obtained by lysing the hemi-embryo and analyzing the thus released DNA after treatment as described by Ninomiy, T. et al. (1989) Molecular Reproduction and Development 1:242-248. Each of the DNA samples is treated with Dpn I. Thereafter, a polymerase chain reaction (Saiki, et al. (1985) Science 230:1350-1354) is preformed to amplify all or part of the transgene. When the entire transgene is amplified, two extension primers each complimentary to opposite strands at opposing ends of the transgene are used for amplification. When, however, less than the entire transgene is amplified, such extension primers are chosen such that the amplified gene product spans the Dpn I site in the transgene. If Dpn I cleavage has not occurred, PCR amplification results in amplified sequences having a predetermined size whereas primer extension for those transgenes which have been cleaved will not result in exponential amplification. Generally, the Dpn I/PCR amplified DNA from the hemi-embryo is subjected to electrophoresis followed by hybridization with labeled probe complimentary to the region of the transgene between the two extension primers. This facilities the determination of the size of the amplified DNA sequences, if any, and provides an indication of whether the transgene has been integrated into the pre-implantation embryo from which the hemi-

embryo was obtained (now called a "transgenic hemi-embryo"). If it has, the remaining untreated transgenic hemi-embryo is transplanted into a recipient parent. After in utero development, the transgenic non-human animal having the desired phenotype conferred by the integrated transgene is identified by an appropriate method in utero or after birth. Of course, other restriction endonucleases capable of cleaving a methylated DNA sequence but incapable of cleaving the unmethylated form of a recognition sequence may be used in the aforementioned method.

The above described method using Dpn I requires that the sequence GATC be present in the transgene of interest. In those cases when such a sequence is not present, it may be readily introduced into the transgene by site directed mutagenesis (Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. 82:488) or cassette mutagenesis (Wells, J.A., et al. (1985) Gene 34:315) provided such mutagenesis does not change the amino acid sequence encoded by the transgene (or causes an inconsequential change in amino acid sequence) and that any codons so generated are functional in the transgenic non-human animal of interest.

The above described methods for the detection of transgenesis in pre-implantation embryos provide economical and time saving method for generating transgenic non-human animals since they significantly decrease the number of pregnancies required to produce a transgenic animal and substantially increase the likelihood that an implanted embryo will produce a transgenic non-human animal. Such methods are especially important for those animals for which very low or non-existent frequencies of transgenesis have been obtained, e.g. bovine species.

In an alternate embodiment, the above described method for detecting transgenesis in pre-implantation embryos is combined with embryonic cloning steps to generate a clonal population of transgenic embryos which may thereafter be implanted into recipient females to produce a clonal population of transgenic non-human animals also having the same genotype. In this regard, it is to be understood that transgenic embryos and/or non-human transgenic animals having the same "genotype" means that the genomic DNA is substantially identical between the individuals of the embryo and/or transgenic animal population. It is to be understood, however, that during mitosis various somatic mutations may occur which may produce variations in the genotype of one or more cells and/or animals. Thus, a population having the same genotype may demonstrate individual or subpopulation variations.

After a hemi-embryo is identified as a transgenic hemi-embryo, it is cloned. Such embryo cloning may be performed by several different approaches. In one cloning method, the transgenic hemi-embryo is cultured in the same or in a similar media as used to culture individual oocytes to the pre-implantation stage. The "transgenic embryo" so formed (preferably a transgenic morula) is then divided into "transgenic hemi-embryos" which can then be implanted into a recipient female to form a clonal population of two transgenic non-human animals. Alternatively, the two transgenic hemi-embryos obtained may be again cultivated to the pre-implantation stage, divided, and recultivated to the transgenic embryo stage. This procedure is repeated until the desired number of clonal transgenic embryos having the same genotype are obtained. Such transgenic embryos may then be implanted into recipient females to produce a clonal population of transgenic non-human animals.

In a preferred cloning method, the transgenic embryo is cloned by nuclear transfer according to the techniques of Prather, et al. (1988) Biol. Reprod. 37:59-86; Roble, et al. (1987) J. Anim. Sci. 64:642-664. According to this method, nuclei of the transgenic embryo are transplanted into enucleated oocytes, each of which is thereafter cultured to the blastocyst stage. At this point, the transgenic embryos may be resubjected to another round of cloning by nuclear transplantation or may be transferred to a recipient parent for production of transgenic offspring having the same genotype.

In addition to the foregoing methods for detecting early transgenesis, other methods may be used to detect transgenesis. Such methods include in utero and post partum analysis of tissue. In utero analysis is performed by several techniques. In one, transvaginal puncture of the amniotic cavity is performed under echoscopic guidance (Bowgso, et al. (1975) Bet. Res. 96:124-127; Rumsey, et al. (1974) J. Anim. Sci. 39:386-391). This involves recovering about 15 to 20 milliliters of amniotic fluid between about day 35 and day 100 of gestation. This volume of amniotic fluid contains about 1000 to 12,000 cells per ml originating from the urogenital tract, the skin and possibly the lungs of the developing embryo. Most of these cells are dead. Such cells, however, contain genomic DNA which is subjected to PCR analysis for the transgene as an indication of a successful transgenesis. Alternatively, fetal cells may be recovered by chorion puncture. This method also may be performed transvaginally and under echoscopic guidance. In this method, a needle is used to puncture the recipient animal's placenta, particularly the placentonal structures, which are fixed against the vaginal wall. Such sampling may be performed around day 60 of gestation in bovine species. Chorion cells, if necessary, are separated from maternal

tissue and subjected to PCR analysis for the transgene as an indication of successful transgenesis.

Transgenesis may also be detected after birth. In such cases, transgene integration can be detected by taking an appropriate tissue biopsy such as from the ear or tail of the putative transgenic animal. About one to two centimeters of tail or about five to ten square millimeters of ear are obtained followed by southern blotting with a probe for the transgene according to the method of Hogan, et al. (1986) Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory.

Transgenesis can also be determined by using the southern blot technique with DNA obtained from other tissues. In particular, semen from a recombinant bull will be useful for identifying transgenic animals.

Transgenesis may also by detected by assaying for expression of the recombinant polypeptide in a tissue, secretion (e.g., saliva), or other body fluid. In the case where the goal is expression of a recombinant polypeptide in milk of cows it will be especially useful to assay the saliva of bulls for expression levels. This is because some mammary specific promoters may also cause salivary gland expression, albeit at low levels. See, e.g., Archibald et al. (1990) Proc. Nat. Acad. Sci. USA 87Z:5178-5182.

In those embodiments where a recombinant polypeptide is expressed and secreted into the milk of transgenic bovine species, the transgenic milk so obtained may be either used as is or further treated to purify the recombinant polypeptide. This depends, in part, on the recombinant polypeptide contained in the transgenic milk and the ultimate use for that protein. Thus, when the recombinant polypeptide is secreted into transgenic milk

to increase the nutritional value of the bovine milk, no further purification is generally necessary. An example of such a situation involves one of the preferred embodiments wherein human lactoferrin is produced in the milk of bovine species as a supplement to control intestinal tract infections in newborn human infants and to improve iron absorption. In other situations, a partial purification may be desired to isolate a particular recombinant polypeptide for its nutritional value. Thus, for example, human lactoferrin produced in transgenic bovine milk may be partially purified by acidifying the milk to about pH 4-5 to precipitate caseins. The soluble fraction (the whey) contains the human lactoferrin which is partially purified.

The recombinant polypeptide contained in bovine transgenic milk may also be used in food formulations. A particularly useful food formulation comprises an infant formula containing one or more recombinant polypeptides from transgenic bovine milk which have either nutritional or other beneficial value. For example, an infant formula containing human lactoferrin from transgenic bovine milk made according to the present invention provides a bacteriostatic effect which aids in controlling diarrhea in newborn. Similarly, recombinant polypeptides such as human casein and human lysozyme may also be generated in transgenic bovine milk to provide nutritional value. Table 2 sets forth the constituents of a typical infant formula. As indicated therein, the protein content varies between about 1.8 and 4.5 grams of protein per 100 kilocalories of formula. Thus, the total protein including recombinant polypeptide should lie between the values at least based on regulatory requirements in the United States from which the formulation in Table 2 is based. The amount of total protein including recombinant polypeptide, of

course, may vary from the foregoing depending upon the local regulations where the particular formula is intended to be used.

Approximately 2 ml of saliva was collected from the mouth of the animal and levels of protein were determined in these samples using a radioimmunoassay as described in Example 5. Of the ten animals, three showed expression of hLF above the lower limit of detection. All three animals were part of the group of four animals judged to be mosaic.

Expression levels were as follows:

animal	sample 1 (ng/ml)	sample 2 (ng/ml)
9772	25	18
9773	3	1.4
9774	1.2	nd

nd = not determined

All 10 animals were also tested for hLZ expression. Only animal 9772 showed expression of hLZ in saliva. The amount detected was 2 ng/ml.

Of the 21 animals born in the experiment described in Example 15, one animal (male) was judged to be mosaic based on the fact that it was immunotolerant for hLF. This animal showed an hLF expression in saliva of 100 ng/ml.

These data show that the transgenes used are capable of expressing hLF (and hLZ) in bovines.

Having described the preferred embodiments of the present invention, it will appear to those ordinarily skilled in the art that various modifications may be made to the disclosed embodiments, and that such modifications are intended to be within the scope of the present invention.

All references disclosed herein are expressly incorporated by reference.

WHAT IS CLAIMED IS:

1. A transgene for producing a recombinant polypeptide in transgenic bovine species comprising at least one expression regulation DNA sequence functional in at least one cell-type of said bovine species operably linked to a recombinant DNA encoding a recombinant polypeptide, wherein said transgene is capable of directing the expression of said recombinant DNA sequence in at least said one cell-type of a bovine species containing said transgene to produce said recombinant polypeptide.

2. The transgene of Claim 1 wherein said expression regulation sequences comprise 5' and 3' expression regulation sequences from a serum albumin, said cell-type is liver cell, said recombinant polypeptide is human serum albumin and said transgene further comprises a secretory DNA sequence functional in said liver cells and operably linked to the recombinant DNA encoding said human serum albumin.

3. A transgene for producing a recombinant polypeptide in the milk of transgenic bovine species comprising at least one expression regulation DNA sequence functional in the mammary secretory cells of said bovine species, a secretory DNA sequence encoding a secretory signal sequence also functional in the mammary secretory cells of said bovine species and a recombinant DNA sequence encoding a recombinant polypeptide, wherein said secretory DNA sequence is operably linked to said recombinant DNA sequence and to form a secretory-recombinant DNA sequence said at least one expression regulation sequence operably linked to said secretory-recombinant DNA sequence, such that said transgene is capable of directing the expression of said secretory-recombinant DNA sequence in mammary secretory cells of

bovine species containing said transgene to produce a form of recombinant polypeptide which when secreted from said mammary secretory cells produces recombinant polypeptide in the milk of said bovine species.

4. The transgene of Claim 1 or 3 further comprising a recombinant intervening sequence.

5. The transgene of Claim 4 wherein said recombinant intervening sequence is a hybrid intervening sequence.

6. The transgene of Claim 5 wherein said hybrid intervening sequence contains a permissive RNA splice signal.

7. The transgene of Claim 3 wherein said recombinant polypeptide is a homologous polypeptide from bovine species.

8. The transgene of Claim 7 wherein said homologous polypeptide is selected from the group consisting of caseins, lactoferrin, lysozyme, cholesterol hydrolase and serum albumin.

9. The transgene of Claim 3 wherein said recombinant polypeptide is a heterologous polypeptide.

10. The transgene of Claim 9 wherein said heterologous polypeptide is selected from the group consisting of human milk proteins, human serum proteins, and industrial enzymes.

11. The transgene of Claim 10 wherein said heterologous polypeptide is a human milk protein.

12. The transgene of Claim 11 wherein said human milk protein is selected from the group consisting of

secretory immunoglobulins, lysozyme, lactoferrin, lactoglobulin, α-lactalbumin and bile salt-stimulated lipase.

13. The transgene of Claim 12 wherein said milk protein is lactoferrin or lysozyme.

14. The transgene of Claim 10 wherein said heterologous polypeptide is a human serum protein.

15. The transgene of Claim 14 wherein said human serum protein is selected from the group consisting of albumin, immunoglobulin, Factor VIII, Factor IX and Protein C.

16. The transgene of Claim 15 wherein said serum protein is albumin.

17. The transgene of Claim 10 wherein said heterologous polypeptide is an industrial enzyme selected from the group consisting of proteases, lipases, chitenases and ligninases.

18. The transgene of Claim 3 wherein said secretory DNA sequence is selected from the group consisting of DNA sequences encoding secretory signal sequences from human lactoferrin, human serum albumin, human lysozyme and secretory signal sequences from bovine αS1-casein, αS2-casein, β-casein, κ-casein, α-lactalbumin, β-lactoglobulin, and serum albumin.

19. The transgene of Claim 18 wherein said secretory DNA sequence is the DNA sequence encoding the signal secretion sequence of bovine αS1 casein.

20. The transgene of Claim 3 wherein said at least one expression regulation sequence comprises 5' expression

regulation DNA sequences operably linked to the 5' end of said secretory-recombinant DNA sequence.

21. The transgene of Claim 20 wherein said 5' expression regulation DNA sequence is selected from the group consisting of 5' expression regulation sequence from bovine genes encoding αS1-casein, αS2-casein, β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin.

22. The transgene of Claim 21 wherein said 5' expression regulation DNA sequence is a proximal 5' expression regulation sequence comprising the promoter of bovine αS1-casein.

23. The transgene of Claim 22 wherein said 5' expression regulation DNA sequence further comprises a distal 5' expression regulation sequence comprising 5'-flanking DNA sequence from bovine αS1-casein.

24. The transgene of Claim 20 further comprising 3' expression regulation sequences operably linked to the 3' end of said secretory-recombinant DNA sequence.

25. The transgene of Claim 24 wherein said 3' expression regulation sequence comprise 3' expression regulation sequence from bovine genes encoding αS1-casein, αS2-casein, β-casein, k-casein, α-lactalbumin, and β-lactoglobulin.

26. The transgene of Claim 25 wherein said 3' expression regulation DNA sequence comprises a 3' proximal expression regulation sequence from bovine αS1-casein.

27. The transgene of Claim 26 wherein said 3' expression regulation DNA sequence further comprises a

3' distal expression regulation sequence from bovine αS1-casein.

28. The transgene of Claim 27 wherein said distal 5' expression regulation DNA sequence comprises about a 30 kb 5'-flanking region of bovine αS1-casein and said distal 3' expression regulation DNA sequence comprises about a 15 kb 3'-flanking region of bovine αS1-casein.

29. A transgenic bovine species capable of producing a recombinant polypeptide in at least one cell type of said animal.

30. A transgenic bovine species capable of producing recombinant polypeptide in the milk of said trangenic species.

31. The transgenic bovine species of Claim 30 wherein said recombinant polypeptide is a homologous polypeptide from bovine species.

32. The transgenic bovine species of Claim 30 wherein said recombinant polypeptide is a heterologous polypeptide.

33. The transgenic bovine species of Claim 32 wherein said heterologous polypeptide is selected from the group consisting of human milk proteins, human serum proteins, and industrial enzymes..

34. The transgenic bovine species of Claim 33 wherein said heterologous polypeptide is a human milk protein.

35. The transgenic bovine species of Claim 34 wherein said human milk protein is selected from the group consisting of secretory immunoglobulins, lysozyme,

lactoferrin, lactoglobulin, α-lactalbumin and bile salt-stimulated lipase.

36. The transgenic bovine species of Claim 35 wherein said milk protein is lactoferrinor lysozyme.

37. The transgenic bovine species of Claim 33 wherein said heterologous polypeptide is a human serum protein.

38. The transgenic bovine species of Claim 37 wherein said human serum protein is selected from the group consisting of albumin, immunoglobulin, Factor VIII, Factor IX and Protein C.

39. The transgenic bovine species of Claim 38 wherein said serum protein is albumin.

40. The transgenic bovine species of Claim 33 wherein said heterologous polypeptide is an industrial enzyme selected from the group consisting of proteases, lipases, chitinases and ligninases.

41. Milk from transgenic bovine species containing a recombinant polypeptide.

42. The milk of Claim 41 wherein said recombinant polypeptide is a homologous polypeptide from bovine species.

43. The milk of Claim 41 wherein said recombinant polypeptide is a heterologous polypeptide.

44. The milk of Claim 43 wherein said heterologous polypeptide is selected from the group consisting of human milk proteins, human serum proteins, and industrial enzymes.

45. The milk of Claim 44 wherein said heterologous polypeptide is a human milk protein.

46. The milk of Claim 45 wherein said human milk protein is selected from the group consisting of secretory immunoglobulins, lysozyme, lactoferrin, lactoglobulin, α-lactalbumin and bile salt-stimulated lipase.

47. The milk of Claim 46 wherein said milk protein is lactoferrin or lysozyme.

48. The milk of Claim 43 wherein said heterologous polypeptide is a human serum protein.

49. The milk of Claim 48 wherein said human serum protein is selected from the group consisting of albumin, immunoglobulin, Factor VIII, Factor IX and Protein C.

50. The milk of Claim 49 wherein said serum protein is albumin.

51. A food formulation comprising transgenic milk containing a recombinant polypeptide.

52. The food formulation of Claim 51 wherein said recombinant polypeptide is at least partially purified from said transgenic milk.

53. The food formulation of Claim 51 formulated with nutrients appropriate for infant formula.

54. A method for producing a transgenic <u>bovine</u> species capable of producing a recombinant polypeptide in the milk of said <u>bovine</u> species, said method comprising:

introducing the transgene of Claim 1 into an embryonal target cell of a bovine species;

transplanting the transgenic embryonal target cell formed thereby or the embryo obtained herefrom into a recipient female bovine parent; and

identifying at least one female offspring which is capable of producing said recombinant polypeptide in the milk of said offspring.

55. A method for producing a transgenic non-human mammal having a desirable phenotype comprising:

(a) methylating a transgene capable of conferring said phenotype when incorporated into the cells of said transgenic non-human animal;

(b) introducing said methylated transgene into fertilized oocytes of said non-human mammal to permit integration of said transgene into the genomic DNA of said fertilized oocytes;

(c) culturing the individual oocytes formed hereby to pre-implantation embryos thereby replicating the genome of each of said fertilized oocytes;

(d) removing at least one cell from each of said pre-implantation embryos and lysing said at least one cell to release the DNA contained therein;

(e) contacting said released DNA with a restriction endonuclease capable of cleaving said methylated transgene but incapable of cleaving the unmethylated form of said transgene formed after integration into and replication of said genomic DNA; and

(f) detecting which of said cells from said pre-implantation embryos contain a transgene which is resistant to cleavage by said restriction endonuclease as an indication of which pre-implantation embryos have integrated said transgene.

56. The method of Claim 55 wherein said removal of at least one cell forms a first and second hemi-embryo for each of said pre-implementation embryos and each of said first hemi-embryos is lysed and analyzed according to steps (d) through (f), said method further comprising;

(g) cloning at least one of said second hemi-embryos; and

(h) to form a multiplicity of transgenic embryos.

57. The method of Claim 56 further comprising transplanting more than one of said transgenic embryos into recipient female parents to produce a population containing at least two transgenic non-human animals having the same genotype.

58. The method of Claim 55 further comprising transplanting the remainder of said pre-implantation embryo containing a genomically integrated transgene into a recipient female parent and identifying at least one offspring having said phenotype.

59. The method of Claim 55 wherein said restriction endonuclease is DPNI and said transgene is methylated at N6 of the adenine of the sequence GATC contained within said transgene.

60. The method of Claim 59 wherein said detection utilizes a polymerase chain reaction using extension primers complementary to sequences upstream and downstream to said GATC sequence.

61. The method of Claim 59 wherein said non-human transgenic mammal is bovine species, said transgene encodes a recombinant polypeptide and said desired phenotype is the ability to produce said recombinant polypeptide in the milk of said bovine species.

56. The method of Claim 55 wherein said removal of at least one cell forms a first and second hemi-embryo for each of said pre-implementation embryos and each of said first hemi-embryos is lysed and analyzed according to steps (d) through (f), said method further comprising;

(g) cloning at least one of said second hemi-embryos; and

(h) to form a multiplicity of transgenic embryos.

57. The method of Claim 56 further comprising transplanting more than one of said transgenic embryos into recipient female parents to produce a population containing at least two transgenic non-human animals having the same genotype.

58. The method of Claim 55 further comprising transplanting the remainder of said pre-implantation embryo containing a genomically integrated transgene into a recipient female parent and identifying at least one offspring having said phenotype.

59. The method of Claim 55 wherein said restriction endonuclease is DPNI and said transgene is methylated at N6 of the adenine of the sequence GATC contained within said transgene.

60. The method of Claim 59 wherein said detection utilizes a polymerase chain reaction using extension primers complementary to sequences upstream and downstream to said GATC sequence.

61. The method of Claim 59 wherein said non-human transgenic mammal is bovine species, said transgene encodes a recombinant polypeptide and said desired phenotype is the ability to produce said recombinant polypeptide in the milk of said bovine species.

62. The method of Claim 61 wherein said transgene is the transgene of Claim 3.

63. A transgene for producing a recombinant polypeptide in the milk of transgenic bovine species comprising:

(i) a bovine 5' expression regulation sequence;

(ii) a secretory DNA sequence encoding a secretory signal sequence functional in the mammary secretory cells of the bovine species;

(iii) a recombinant DNA sequence encoding a recombinant polypeptide, said secretory DNA sequence being operably linked to said recombinant DNA sequence, wherein a secretory-recombinant DNA sequence is formed, said secretory-recombinant DNA sequence being operably linked to said bovine expression regulation sequence;

(iv) a 3' untranslated sequence;

(v) a 3' flanking sequence of a bovine gene; and

wherein said transgene is capable of directing the expression of said secretory-recombinant DNA sequence in mammary secretory cells of bovine species containing said transgene to produce a form of recombinant polypeptide which when secreted from said mammary secretory cells produces recombinant polypeptide in the milk of said bovine species.

64. The transgene of claim 63, further comprising a recombinant intervening sequence.

65. The transgene of claim 64 wherein the recombinant intervening sequence is a hybrid intervening sequence.

66. The transgene of claim 65 wherein the hybrid intervening sequence comprises a 5' portion of an intervening sequence from bovine α-S_1-casein and a 3'

sequence portion of an IgG heavy chain intervening sequence.

67. The transgene of claim 66 wherein the 3' sequence portion is a 3' splice signal sequence associated with the J-C segment rearrangement of an IgG heavy chain.

68. The transgene of claim 63, wherein the bovine expression regulation sequence and the 3' flanking sequence are derived from the same bovine gene.

69. The transgene of claim 63, wherein the bovine expression regulation sequence, the 3' untranslated sequence, and the 3' flanking sequence are derived from the same bovine gene.

70. The transgene of claim 68 or claim 69 wherein the bovine gene is α_{S_1}-casein.

71. The transgene of Claim 70 wherein the bovine expression regulation sequence comprises about a 30kb 5'-flanking region of bovine αS1-casein and the 3'-flanking sequence comprises about a 15kb 3'-flanking region of bovine αS1-casein.

72. The transgene of claim 3 or claim 63 wherein the milk comprises greater than 50 micrograms of the recombinant polypeptide per milliliter.

73. A transgenic bovine species capable of producing a recombinant polypeptide in saliva.

74. The semen of a transgenic bovine.

75. A transgene for producing a recombinant polypeptide in the milk of transgenic bovine species comprising:

(i) a 5' expression regulation sequence;

(ii) a secretory DNA sequence encoding a secretory signal sequence functional in the mammary secretory cells of the bovine species;

(iii) a recombinant DNA sequence encoding a recombinant polypeptide, said secretory DNA sequence being operably linked to said recombinant DNA sequence, wherein a secretory-recombinant DNA sequence is formed, said secretory-recombinant DNA sequence being operably linked to the 5' expression regulation sequence;

(iv) a 3' untranslated sequence; and,

(v) a 3' flanking sequence from a human gene;

wherein the transgene is capable of directing the expression of the secretory-recombinant DNA sequence in mammary secretory cells of bovine species containing the transgene

to produce a form of recombinant polypeptide which when secreted from the mammary secretory cells produces recombinant polypeptide in the milk of the bovine species.

76. The transgene of claim 75, wherein the 5' expression regulatory sequence is a bovine sequence.

77. The transgene of claim 75 or claim 76, wherein the 3' flanking sequence is from the human lactoferrin (hLF) gene.

78. The transgene of claim 77, wherein the 3' flanking sequence is 9 kilobase pairs in length.

79. The transgene of claim 77, wherein the recombinant polypeptide is human lactoferrin.

80. The transgene of claim 75, wherein the 5' expression regulation sequence, the secretory DNA sequence, the recombinant DNA sequence encoding a

recombinant polypeptide, the 3' untranslated sequence; and the 3' flanking sequence are from a human gene.

81. The transgene of claim 80, wherein the human gene is the lactoferrin gene.

82. A method for expressing a human polypeptide in the milk of a bovine comprising:

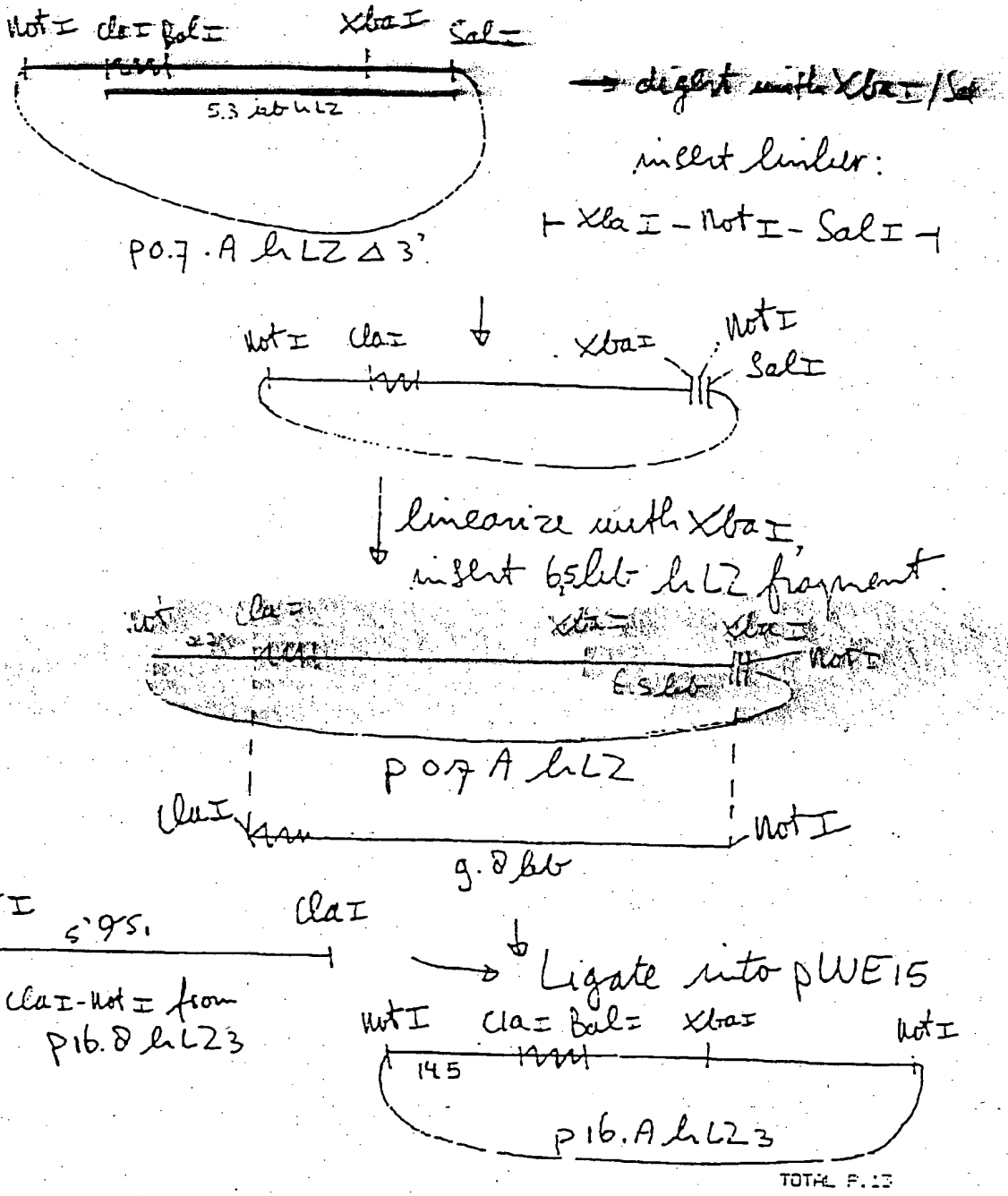
introducing a human genomic fragment encoding the human polypeptide into an embryonal target cell of a bovine species;

transplanting the transgenic embryonal target cell formed thereby or the embryo obtained therefrom into a recipient female bovine parent; and

identifying at least one female offspring which is capable of producing the recombinant polypeptide in the milk of the offspring.

83. The method of claim 82, wherein the human polypeptide is lactoferrin.

Construction of 16.A hLZ 3: Fig. 27



TOTAL P.12

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Dairy Sci., Volume 72, Issued 1989, Bremed et al., "Alteration of milk composition using molecular genetics", pages 2826-2833, see the entire document.	1-28,63-72,75-81
Y	Theriogenology, Volume 25, No. 1, Issued January 1986, Loskutoff et al., "Gene microinjection of bovine embryos facilitated by centrifugation", page 168, see the entire abstract.	1-28,63-72,75-81
Y	Theriogenology, Volume 29, No. 1, issued January 1988, Biery et al., "Gene transfer by pronuclear injection in the bovine", page 224, see the entire abstract.	1-28,63-72,75-81

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

DIALOG (Files 154, 55, 311, 312)
U.S. Automated Patent System (File USPAT, 1975-1993).
Search terms: transgene, transgenic, cow, milk, bovine, albumin, casein, lactoferrin, mammary, liver, secretory, inventors' names.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claims 1-28, 63-72 and 75-81, drawn to a transgene, classified in Class 536, subclasses 27, for example.

Group II, claims 29-40, 54, 82 and 83, drawn to a transgenic bovine species and a method of making the same, classified in Classes 800 and 435, subclasses 2 and 172.3 respectively, for example.

Group III, claims 41-53, drawn to milk from a transgenic bovine species, classified in Class 426, subclass 580, for example.

Group IV, claims 55-62, drawn to method of producing a transgenic non-human mammal having a desired phenotype, classified in Class 800, subclass 2, for example.

Group V, claim 73, drawn to a transgenic bovine species, classified in Class 800, subclass 2, for example.

Group IV, claim 74, drawn to semen of a transgenic bovine, classified in Class 435, subclass 2, for example.

Groups I, II, III, V and VI are each directed to a different and independent product. Group IV is directed to a method of producing a transgenic non-human mammal with a desired phenotype. The method of Group V is not required to produce the transgenic bovine of either Group II or V. Accordingly, the claims are not so linked by a "special technical feature" within the meaning of PCT Rule 13.2 as to form a single inventive concept. See PCT Rule 13.2(i).